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Sialidase Fusion Protein as a Novel Broad-Spectrum Inhibitor of Influenza Virus Infection

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Influenza is a highly infectious disease characterized by recurrent annual epidemics and unpredictable major worldwide pandemics. Rapid spread of the highly pathogenic avian H5N1 strain and escalating human infections by the virus have set off the alarm for a global pandemic. To provide an urgently needed alternative treatment modality for influenza, we have generated a recombinant fusion protein composed of a sialidase catalytic domain derived from *Actinomyces viscosus* fused with a cell surface-anchoring sequence. The sialidase fusion protein is to be applied topically as an inhalant to remove the influenza viral receptors, sialic acids, from the airway epithelium. We demonstrate that a sialidase fusion construct, DAS181, effectively cleaves sialic acid receptors used by both human and avian influenza viruses. The treatment provides long-lasting effect and is nontoxic to the cells. DAS181 demonstrated potent antiviral and cell protective efficacies against a panel of laboratory strains and clinical isolates of IFV A and IFV B, with virus replication inhibition 50% effective concentrations in the range of 0.04 to 0.9 nM. Mouse and ferret studies confirmed significant in vivo efficacy of the sialidase fusion in both prophylactic and treatment modes.

Influenza, caused by infection with influenza virus A (IFV A) and IFV B, carries enormous direct and indirect socioeconomic impacts. Since 1997, a new avian IFV A virus of the H5N1 type has been causing epidemics in wild birds, as well as in domestic poultry. Alarmingly, human infections by this virus are also on the rise. Thus far, >100 people have been confirmed to be infected by the virus and >50 of them have died (5). Evidence has also shown that since 1999, the H5N1 virus has been evolving rapidly in ducks and has become increasingly pathogenic in both chicken and mice (7). All of these are serious warning signs that a pandemic may be imminent. No vaccine is currently available against the future pandemic virus. The neuraminidase inhibitor (NAI), oseltamivir, was linked to a surprisingly high frequency of drug-resistant viruses in children (26). Oseltamivir-resistant H5N1 virus has also been isolated from a patient who had been taking the drug as a prophylactic measure (27). For these reasons, it is imperative to develop alternative approaches to prevent and treat influenza.

The host cell receptors for influenza A and B viruses are cell surface sialic acids (20). The predominant type of sialic acids is *N*-acetylneuraminic acid (Neu5Ac), which is the biosynthetic precursor for most of the other types. In nature, Neu5Ac is mostly linked to the penultimate galactose residues of carbohydrate side chains via α(2,3)- or α(2,6)-linkages. Both Neu5Ac α(2,3)-Gal and Neu5Ac α(2,6)-Gal molecules can be recognized as a receptor by influenza viruses (44), but human viruses prefer α(2,6)-linked sialic acid, whereas avian and equine viruses predominantly recognize α(2,3)-linked sialic acid (20). The human respiratory epithelium expresses both forms of sialic acids, but α(2,6)-linked sialic acid is more abundant than α(2,3)-linked sialic acid (15, 29). This explains the fact that avian influenza viruses can infect humans, although the infection is very inefficient. The relatively low abundance of α(2,3)-linked sialic acid in human airway epithelium in large part causes the species barrier for avian viruses. This demonstrates that merely reducing the sialic acid level on the airway surface would have significant impact on IFV infectivity to humans.

To render the target cells inaccessible to influenza viruses, we generated a novel recombinant fusion protein consisting of a sialidase fused with a respiratory epithelium-anchoring domain. Sialidases, also referred to as neuraminidases, are a family of exoglycosidases that catalyze the removal of terminal sialic acid residues from various glycoconjugates, such as glycoproteins and glycolipids. Sialidases were previously demonstrated to be effective inhibitors of influenza virus infection in vitro. Even before sialic acid was proven to be the receptor for influenza viruses, it was observed that when sialic acid was enzymatically removed from the cell surface, the cells were less susceptible to infection by influenza viruses (12). In several experiments performed much later, MDCK (Madin-Darby canine kidney) or EAC (Ehrlich ascites carcinoma) cells were briefly treated with *Vibrio cholerae* sialidase and then infected with influenza virus. Influenza virus infections were decreased by 90 to 100% as a result of the sialidase treatment (6, 13, 52). *Micromonospora viridifaciens* sialidase was also used to destroy cellular influenza virus receptors in cell culture assays (2). In another case, influenza virus NA, which is also a sialidase, was expressed in CV-1 cells by vaccinia virus. The cells expressing

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the flu NA were resistant to subsequent influenza virus infections (9).

More than 15 sialidase proteins have been purified from microbes and higher eukaryotes. They vary greatly from one another in substrate specificity and enzyme kinetics. Among them, the large bacterial sialidases tend to be the more robust enzymes (1, 8, 11). Among the large bacterial sialidases, such as the ones from *Arthrobacter ureafaciens*, *Clostridium perfringens*, *V. cholerae*, and *Actinomyces viscosus*, the catalytic domain of *A. viscosus* sialidase was selected as the sialidase component of the novel therapeutic candidate for influenza based on three criteria: (i) *A. viscosus* sialidase has broad substrate specificity (54); (ii) it has one of the highest specific activities reported (54); and (iii) it should be well tolerated by the human immune system because *A. viscosus* is a part of the normal oral and gastrointestinal flora in humans (53) which normally exposes the human mucosal surface to the sialidase.

Because influenza viruses primarily infect the upper and central respiratory tract, the sialidase fusion protein will be delivered as an inhalant in humans. However, retention of drug molecules delivered to the respiratory mucosa is generally short due to the mucociliary clearance mechanisms. We reasoned that an epithelium-anchoring domain would tether the sialidase to the respiratory epithelium and increase its retention time and potency. We use the heparin-binding sequence derived from the human protein amphiregulin (AR) (46) as the epithelium-anchoring domain because of its high affinity to heparin and its ability to bind to the glycosaminoglycans (GAGs) present on the respiratory epithelial surface. Here we present data demonstrating potent protective effect of the novel sialidase fusion protein against a spectrum of influenza viruses both in vitro and in vivo.

MATERIALS AND METHODS

Cloning of the sialidase catalytic domain/amphiregulin GAG binding sequence fusion proteins. AvCD-AR (DAS181) consists of a heparin-binding domain derived from amphiregulin (amino acid residues 125 to 145 in GenBank entry AAH09799) fused via its N terminus to the catalytic domain of *A. viscosus* sialidase (AvCD, amino acid residues 274 to 667 in GenBank entry X62276) (59). The boundaries of catalytic domain were determined based on structural homology to enzymes of *M. viridifaciens* and *Salmonella enterica* serovar Typhimurium (PDB accession numbers 1EUR and 2SIM [www.rcsb.org/pdb]). A three-dimensional homology model was built by using the Swiss-Model software (www.expasy.org/swissmod). The DNA fragment coding for AvCD-AR was cloned into pTrc99a vector (Pharmacia) under the control of IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. In another construct AR-AvCD (DAS178), the AR sequence was fused via its C terminus of the AvCD domain. Two control constructs, DAS180 and DAS185, were also made. To make DAS180, the AR sequence in DAS178 was deleted and a N-terminal His_6 tag was added. DAS185 differs from DAS181 in amino acid alteration (Y348F) at the sialidase reactive center that resulted in a >400 times reduction in sialidase activity (data not shown).

Protein expression, purification, and activity assay. The DAS181, DAS178, or DAS185 constructs were expressed in the BL21 strain of *Escherichia coli*. Cells were lysed by sonication in 50 mM phosphate buffer (pH 8.0), 0.3 M NaCl, and 10% glycerol. Clarified lysate was passed through an SP-Sepharose column. Proteins were eluted from the column with lysis buffer that contained 0.8 M NaCl. The fraction eluted from SP-Sepharose was adjusted to 1.9 M $(NH_4)_2SO_4$, clarified by centrifugation, and loaded onto a butyl-Sepharose column. The column was washed with 2 volumes of 1.3 M $(NH_4)_2SO_4$, and the fusion protein was eluted with 0.65 M $(NH_4)_2SO_4$. For the final step, size exclusion chromatography was performed on Sephacryl S-200 equilibrated with phosphate-buffered saline (PBS). Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reversed-phase high-pressure liquid chromatography, and enzyme-linked immunosorbent assay with the antibodies generated against *E. coli* cell proteins and estimated to be >98% (not shown). For the purification of DAS180 (His_6-AvCD), cation exchange on SP-Sepharose was replaced with metal chelate affinity chromatography on nickel-charged chelating Sepharose (Amersham). All buffers remained the same, except that elution was performed with 0.25 M imidazole in lysis buffer.

Neu2 was expressed and purified as previously described (34). *C. perfringens* and *A. ureafaciens* sialidases were purchased from Sigma (St. Louis, MO) and Prozyme (San Leandro, CA), respectively.

The sialidase activity was measured by using the fluorogenic substrate 4-MU-NANA (4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid; Sigma). One unit of sialidase is defined as the amount of enzyme that releases 10 nmol of MU from 4-MU-NANA in 10 min at 37°C (50 mM CH_3COOH-NaOH buffer [pH 5.5]) in a reaction that contains 20 nmol of MU-NANA in a 0.2-ml volume (38). The protein concentration was determined by using Bio-Rad's Bradford kit. The specific activity of AvCD-AR was 1,300 U/mg of protein (0.77 μg of DAS181 protein per unit of activity).

Cell surface sialic acid detection. Confluent monolayers of MDCK cells in 96-well plates were treated with 100 mU (0.1 ml total volume) of DAS181 or DAS180/well for 1 or 2 h at 37°C. The cells were then washed three times with PBS and either fixed immediately with 0.05% glutaraldehyde in PBS or chased for various times with growth media and then fixed. Levels of α(2,6)-linked sialic acid, α(2,3)-linked sialic acid, or total sialic acid were detected by using a cell-based enzyme-linked lectin assay (23) with minor modifications. The fixed cells were blocked with 3% bovine serum albumin (BSA) in PBS and streptavidin-biotin blocking reagent (Vector Laboratories, Burlingame, CA) to block endogenous streptavidin- and biotin-binding sites. Cells were rinsed once with PBS–0.1% Tween 20 (PBST) and incubated with either 2 μg of biotinylated SNA lectin (Vector Laboratories)/ml, 20 μg of biotinylated MAA lectin (Vector Laboratories)/ml, or 20 μg of biotinylated LFA lectin (EY Laboratories, San Mateo, CA)/ml for 2 h at 37°C. SNA (*Sambucus nigra*) is specific for Neu5Ac α(2,6)-Gal, MAA (*Maackia amurensis*) is specific for Neu5Ac α(2,3)-Gal, and LFA (*Limax flavus*) is specific for sialic acids. The cells were washed four times in PBST. Secondary detection of the bound lectin was accomplished by incubating the cells with 5 μg of streptavidin-HRP (streptavidin conjugated with horseradish peroxidase; Vector Laboratories)/ml for 1 h at 37°C. Cells were washed five times in PBST, developed in tetramethyl benzidine (TMB; Sigma), and stopped in 1 M H_2SO_4. The absorbance was measured at 450 nm, and the percentage of sialic acid remaining was calculated by using the following calculation: 100% × [(absorbance of treated cells − background)/(absorbance of vehicle-treated cells − background)]. Wells treated with streptavidin-HRP alone without the lectins were the background controls.

IFV binding to MDCK and fetuin-coated plates. A/PR/8/34 was biotinylated with EZ-link Sulfo-NHS-SS-Biotin (Pierce). 100 μl of fetuin (10 mg/ml) was adsorbed to a 96-well plate overnight and blocked with 3% BSA for 30 min at 37°C. Confluent MDCK monolayers in 96-well plates (5.75×10^5 cells per well) and the fetuin-coated plates were treated with either binding medium (Dulbecco modified Eagle medium–F-12 [DMEM:F12] plus 0.2% BSA) alone or 5 U of DAS181/ml in binding medium for 2 h at 37°C. After the incubation, the plates were washed two times with ice-cold PBS, and the plates were chilled further for 30 min at 4°C. The input virus was diluted in binding medium on ice, added to the appropriate wells, and incubated for 90 min at 4°C. Cells were washed three times with chilled PBS to remove unbound virus, fixed with 0.05% glutaraldehyde, incubated with streptavidin-HRP, and developed by using TMB. Wells without the virus were included for background streptavidin-HRP binding.

Cell protection assay. All laboratory IFV A and B strains were obtained from the American Type Culture Collection (Manassas, VA) with the exception of A/turkey/Wisconsin/66, A/PR/8/34, A/Japan/305/57, and A/Victoria/504/2000, which were from Charles River Laboratories. The low-passage 2004 IFV clinical virus isolates (A/New Caledonia/20/99, A/Panama/2007/99, and B/Hong Kong/330/01) were generously provided by Alexander Klimov, Centers for Disease Control and Prevention. The A/gull virus was obtained from Robert Webster, St. Jude Children's Research Hospital (Memphis, TN). For cell protection assays, quadruplicate MDCK cell monolayers in microplate wells were treated with various dilutions of DAS181 in EDB-BSA (10 mM sodium acetate [pH 6.0], 0.1 M NaCl, 10 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.5% BSA) at 37°C for 2 h. Influenza viruses (multiplicity of infection [MOI] of 0.01) were added to both the sialidase-treated cells and the control cells treated with the enzyme dilution buffer only. After 1 h, the cells were washed with PBS three times and incubated at either 37 or 35°C in DMEM:F12 supplemented with 0.2% ITS (insulin-transferrin-selenium; Invitrogen, Carlsbad, CA) and 0.3 μg of acetylated trypsin (Sigma)/ml. After 72 h, the cells were stained with 0.5% crystal violet in 20% methanol for 5 min, rinsed with tap water, and dried. The level of viable cells in each well was quantitated by extracting crystal violet with 70% ethanol and reading the absor-

bance at 570 nm. The percentage of cell protection was calculated by using the following formula: 100 × [(sialidase-treated sample − virus only)/(uninfected sample − virus only)].

Viral replication inhibition assay. Quadruplicate MDCK monolayers in 96-well plates were treated with various dilutions of DAS181 in EDB-BSA buffer for 2 h at 37°C. Both the sialidase-treated cells and the untreated control cells (treated with only EDB-BSA buffer) were infected with a virus MOI of 0.01. After 30 min, the cells were washed two times with PBS and incubated at either 37 or 35°C in DMEM:F12 supplemented with 0.2% ITS and 0.3 μg of acetylated trypsin (Sigma, St. Louis, MO)/ml. At 40 to 48 h postinfection, the cells were fixed in 0.05% glutaraldehyde in PBS. They were then blocked in 3% BSA in PBS for 30 min at 37°C (or 4°C overnight). Cells were washed once with PBST. Each well was incubated with 50 μl of either 6.8 μg of anti-IFV A NP monoclonal antibody or 7.2 μg of anti-IFV B NP antibody (Fitzgerald Industries, Concord, MA)/ml for 2 h at 37°C. The excess primary antibody was washed away with four washes of PBST. Each well was then incubated with 50 μl of a 1:5,000 dilution of protein G conjugated to HRP (Sigma). Excess secondary reagent was removed by washing the plates five times with PBST. Plates were developed by incubating each well with 50 μl of TMB substrate (Sigma) and were stopped by the addition of 50 μl of 1 M H_2SO_4. The absorbance was measured at 450 nm. Wells containing uninfected cells were used as the background control.

Mouse studies. The experiments were conducted according to the protocol approved by the Animal Care and Use Committee and conducted at the Laboratory of Animal Research Center at Utah State University, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Female BALB/c mice (18 to 21 g) were obtained from Charles River Laboratories (Wilmington, MA) and were maintained on Wayne Lab Blox and tap water ad libitum. The animals were quarantined for 24 h prior to use. Influenza virus A/NWS/33 (H1N1) was originally obtained from Kenneth Cochran of the University of Michigan (Ann Arbor, MI). It was passaged in MDCK cells and pretitrated in mice prior to use in the experiments. Arterial oxygen saturation (SaO_2) was determined by using the Ohmeda Biox 3800 pulse oximeter (Ohmeda, Louisville, OH). The ear probe attachment was used, and the probe was placed on the thigh of the animal. Readings were made after a 30-s stabilization time for each animal. Use of an earlier Ohmeda model (model 3740) for measuring effects of influenza virus on SaO_2 in mice has been previously described (48). To determine lung virus titer, each mouse lung was homogenized, and various dilutions were assayed in triplicate for infectious virus in MDCK cells as described previously (47). Each lung homogenate was centrifuged at 2,000 × g for 5 min, and the supernatants were used in these assays. Increases in the numbers of total survivors were evaluated by chi-square analysis with Yates' correction. Increases in the mean day to death, differences in mean SaO_2 values, the mean lung weight, and mean virus titers were analyzed by using a two-tailed *t* test.

In all three studies, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) prior to treatment with DAS181 or placebo and prior to virus inoculation. DAS181 or placebo was given intranasally in a 50-μl volume at each treatment using various regimens as described above. Infection was induced by intranasal inoculation of A/NWS/33 at a 100% lethal dose (LD_{100}) (i.e., 200 PFU per mouse). The infectious dose was based on previous titration results. Apparently, the virus used in the second study was less virulent than expected. The virus was then retitrated, and the newly determined LD_{100} was used in the third study. Generally, 22 mice were used in each treatment group, and 40 were used in the placebo group. Ten mice in each infected, treated group (20 mice in the placebo group) were observed for 21 days for survival; 3 additional mice from each group were sacrificed on days 1, 3, 6, and 9 for assignment of lung score and for the determination of lung weight and lung virus titer. At each time point, three normal control mice were also sacrificed to provide background data.

Ferret study design and methods. The experiment was conducted according to the protocol approved by the Animal Care and Use Committee and conducted at the Center for Comparative Medicine at the University of Virginia, which is accredited by the AAALAC.

Young female ferrets (0.5 to 0.8 kg) (Marshall Farms, North Rose, NY) were allowed to acclimate for 3 days before the experiment. A preparation of DAS178 dissolved in PBS that contains 500 U/ml in sialidase activity was used in the study. Animals in the AR-AvCD treatment groups received 1 ml of AR-AvCD solution at each dose. Ferrets were anesthetized (20 mg of ketamine/1 mg of xylazine per kg, given intramuscularly) and inoculated intranasally (0.5 ml into each nostril) with DAS178 or PBS twice daily (8 a.m. and 6 p.m.) for a total of 7 days (2 days prior to the viral challenge and 5 days after virus inoculation). The ferrets were observed after the drug application for signs of intolerance. Viral inoculation was carried out on day 3 between 10 and 11 a.m. The viral challenge was carried out using human A/Bayern/7/95(H1N1)-like virus at a 50% tissue culture infective dose of 10^5 (50% inhibitory concentration of $\geq 10^4$ in ferrets). The nasal washes were collected from all animals starting day 2 after DAS178 treatment and continued until day 7.

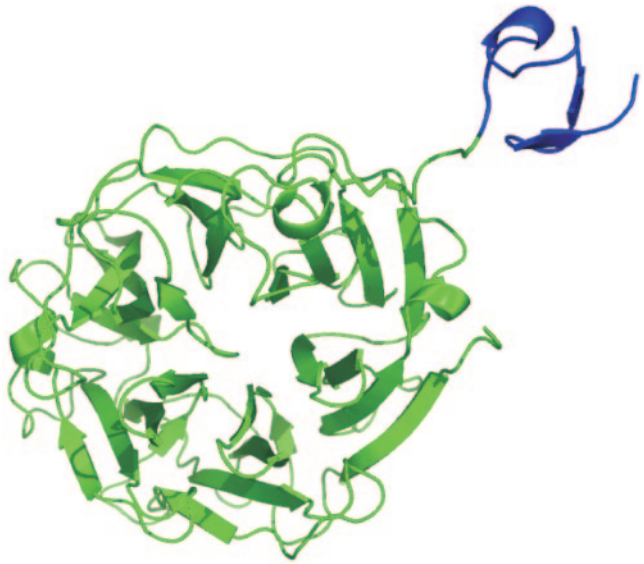

FIG. 1. Molecular model of DAS181. The catalytic domain of the sialidase (AvCD) is colored in green and the protruding anchoring domain (AR) on C terminus in blue. The model was built using the SWISS-MODEL software.

To collect nasal washes, 1 ml of sterile PBS was administered intranasally, the sneezed liquid was harvested, and its volume was recorded. The nasal washes were centrifuged. The pelleted cells were resuspended and counted in a hemacytometer under a microscope. The supernatants were collected, divided into aliquots, and stored at −80°C. The protein concentration in cell-free nasal washes was determined with a protein reagent from Bio-Rad (Hercules, CA). For virus titration of the nasal washes, inoculated MDCK cells were incubated for 3 days at 36°C in a CO_2 incubator. The monolayers were inspected visually for cytopathic effect, and aliquots of the cell culture supernatants from each well were tested for the presence of virus by a standard hemagglutination assay using guinea pig red blood cells. The virus titer was determined by the Spearman-Karber method (18).

RESULTS

Construction, purification, and characterization of DAS181. After many experiments, we chose the *A. viscosus* sialidase catalytic domain and the GAG-binding sequence in human AR as the two basic components of the therapeutic candidates. Most of the data presented here were obtained with the AvCD-AR construct, referred to as DAS181, in which the AR sequence is fused with AvCD at the C terminus (Fig. 1). DAS181 is expressed in *E. coli* and purified to homogeneity. The purified DAS181 protein has an approximate molecular mass of 44,800 Da. The specific activity of DAS181 (1300 U/mg) is more than 100 times higher than that of the human sialidase Neu2 fusion protein (8 U/mg) (34) and more than two times higher than that of *C. perfringens* (333 U/mg) or *A. ureafaciens* (82 U/ml). DAS181 is soluble at concentrations of >50 mg/ml and in 2 M ammonium sulfate. It remains stable at pH 4.5 to 10. No loss of activity for DAS181 was detected after 8 months at 25 or 4°C. DAS181 was incubated with human respiratory mucus at 37°C for 7 days and maintained full activity (data not shown).

DAS181 effectively removes both α(2,3)- and α(2,6)-linked

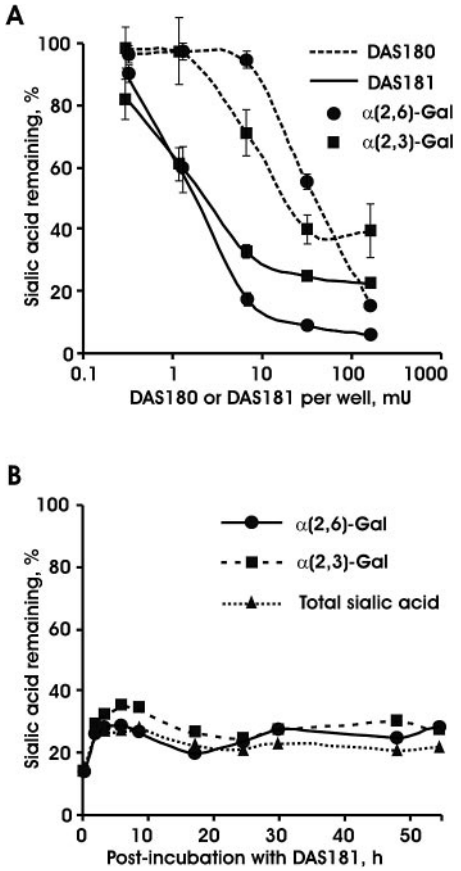

FIG. 2. Sialic acid removal and turnover on MDCK cells. (A) Levels of α(2,6)- and α(2,3)-linked sialic acids and sialic acids in total on the surface of MDCK cells that were pretreated with vehicle (EDB-BSA) or various dilutions of DAS180 (His_6-AvCD) or DAS181 (AvCD-AR) in the vehicle for 1 h. (B) Level of cell surface sialic acid after a single treatment by DAS181. MDCK cells in confluent monolayers were treated with 100 mU of DAS181 for 2 h, washed, and chased for various times with fetal bovine serum-containing medium. The levels of sialic acids were detected with biotinylated lectins. The error bars indicate one standard deviation above or below the mean of three samples.

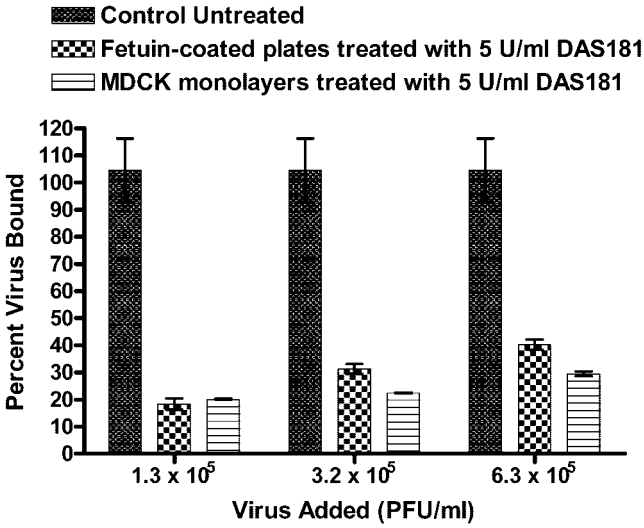

FIG. 3. IFV binding to MDCK and fetuin-coated plates. Biotinylated A/PR/8/34 was allowed to bind to the DAS181-treated fetuin or MDCK monolayers for 30 min at 4°C. The bound virus was detected by using streptavidin-HRP and developed by using TMB. Virus binding to the untreated MDCK cells represented 100%. Wells without the virus were included for background streptavidin-HRP binding. The error bars indicate one standard deviation above or below the mean of three samples.

sialic acid from the cell surface (Fig. 2). As measured by the 50% effective concentration (EC_{50}) for sialic acid removal, DAS181 was more than 10 times more effective than its counterpart (DAS180, His_6-AvCD) that lacks the AR tag (Fig. 2A), demonstrating that the AR tag increases the potency of the sialidase. In MDCK cells, the cell surface sialic acid level remains low and essentially unchanged for at least 2 days after a single treatment by DAS181 (Fig. 2B); the surface sialic acid level in the DAS181 treated MDCK cells rebounded to more than 80% of the normal level after 80 h (data not shown).

Antiviral activity of DAS181 in vitro. Consistent with the proposed mechanism of action for the sialidase fusion protein, the binding of A/PR/8 (H1N1) to DAS181-treated MDCK cells and fetuin (a sialylated protein isolated from fetal bovine serum)-coated wells was greatly diminished (Fig. 3). The extent of binding inhibition correlates very well with the degree of sialic acid removal (data not shown).

DAS181 anti-IFV activity was evaluated in vitro by viral inhibition and cell protection assays in MDCK cells. DAS181 demonstrated potent efficacy against a panel of laboratory strains and recent clinical isolates of influenza A and B viruses in cell protection and/or virus inhibition assays (Tables 1 and 2). These data were further confirmed, with similar EC_{50} values between 1.7 and 13.7 nM, by an experiment performed at a separate facility with four additional IFV strains (A/New Caledonia/20/99 [H1N1], A/Panama/2007/99 [H3N2], A/gull/PA/4175/83 [H5N1], and B/Hong Kong/330/01). DAS181 maintained similar levels of potency even when the IFV infectious dose was increased by 100 times or higher (Table 3). In support of the slow turnover rate of cell surface sialic acid, treatment of cells 24 h prior to the viral challenge protected the cells to the same degree as treating the cells immediately before the challenge (not shown). The anti-IFV activity of DAS181 was also compared to both DAS180 and DAS185, an inactive mutant of DAS181. Based on the cell protection EC_{50}, DAS181 was 5- to 30-fold more potent than DAS180 (data not shown), which again demonstrated that the AR sequence increases treatment potency of the sialidase. As expected, DAS185 provided no protection against IFV (not shown).

The cell protection by DAS181 was further evaluated under various experimental protocols that differed in the following aspects: incubation time with DAS181, washing steps, order of addition of the virus, and whether DAS181 was incubated with cells during the entire course of the infection (Table 4). Variation of the infection protocols altered the EC_{50} values to various degrees. Protocol P1 represents our standard cell protection and inhibition of viral replication assay condition. The three protocols that yielded the lowest EC_{50} values—P3, P4, and P6—represent conventional assay conditions for testing antiviral chemical compounds in which the drug candidate was available throughout the infection. Noticeably, in situations

TABLE 1. Inhibition of IFV replication and cell protection by DAS181[a]

Virus	Mean inhibition of viral replication ± SD		Mean cell protection ± SD	
	EC_{50} (nM)	EC_{90} (nM)	EC_{50} (nM)	EC_{90} (nM)
A/PR/8/34 (H1N1)	0.8 ± 0.9‡	3.2 ± 4.7‡	1.1 ± 0.6*	12.9 ± 9.7†
A/WS/33 (H1N1)	0.4 ± 0.6*	1.1 ± 1.4*	0.2 ± 0.1*	32.7 ± 11.1*
A/NWS/33 (H1N1)	ND	ND	0.4 ± 0.3‡	11.5 ± 19.6*
A/Weiss/43 (H1N1)	0.9 ± 1.6‡	1.7 ± 1.8‡	15.6 ± 16.9§	37.0 ± 40.0*
A/Denver/1/57 (H1N1)	0.5 ± 0.6‡	7.0 ± 12.3*	3.2 ± 4.3§	14.5 ± 14.9*
A/Japan/305/57 (H2N2)	0.3 ± 0.3*	4.7 ± 0.4*	0.1 ± 0.1*	0.2 ± 0.2‡
A/Victoria/504/2000 (H3N2)	0.4 ± 0.6*	4.7 ± 1.9*	1.9 ± 1.9‡	6.5 ± 86.8†
A/Hong Kong/8/68 (H3N2)	0.1 ± 0.1‡	4.4 ± 1.9¶	14.3 ± 23.1§	50.4 ± 37.6†
A/Port Chalmers/1/73 (H3N2)	0.05 ± 0.02†	0.4 ± 0.2‡	2.4 ± 3.2‡	17.7 ± 14.0†
A/Victoria/3/75 (H3N2)	0.1 ± 0.1‡	2.2 ± 4.3¶	2.6 ± 4.2‖	5.1 ± 5.2‡
B/Lee/40	0.3 ± 0.2¶	0.8 ± 0.4§	0.4 ± 0.5*	3.1 ± 4.0*
B/Maryland/1/59	0.04 ± 0.01†	0.7 ± 0.3*	0.5 ± 0.2†	9.8 ± 13.6¶
A/turkey/Wis/66 (H9N2)	0.4 ± 0.1‡	0.8 ± 0.4*	0.1 ± 0.1¶	3.7 ± 4.0*
A/equine/Prague/1/56 (H7N7)	ND	ND	0.2 ± 0.2†	2.8 ± 3.2*

[a] The EC_{50} (or EC_{90}) is the concentration of DAS181 that inhibited viral replication by 50% (or 90%) or that gave rise to 50% (or 90%) cell protection. A value of 1 nM DAS181 equals 45 ng/ml of the protein. Experiments were repeated at least three times, and the actual number of repetitions is indicated as follows: †, 3; *, 4; ‡, 5; ¶, 6; §, 7; and ‖, 8). The error is the standard deviation of these measurements. ND, not determined.

where DAS181 was removed during the remainder of the infection time (P1, P2, and P5), cells were still protected from IFV infection despite higher EC_{50} values. Interestingly, under the similar experimental conditions, oseltamivir was inactive (50).

At the maximum feasible concentration (1,000 U/ml), DAS181 did not affect cell growth curves over a period of 10 days for A549, CACO-2, and MDCK cells (data not shown). In the HAE (human airway epithelium) cultures (49, 60), which closely mimic the native human airway epithelium, DAS181 treatment did not cause cell death, nor did it result in significant changes in the production of gamma interferon, interleukin-1α (IL-1α), IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor alpha over a 7-day incubation period during which fresh DAS181 was added daily (data not shown). These data indicated that DAS181 treatment would not be cytotoxic to the human airway epithelium.

Prevention and treatment of influenza in vivo. We used a murine model of influenza virus infection (47) to assess the antiviral activity of DAS181 in vivo. Study 1 (see Table 5) demonstrated striking efficacy of DAS181 on the survival of the infected animals when given as a prophylaxis over a very broad dose range. The DAS181 treatment also significantly improved lung function and lung pathology, as measured by lung weight, and markedly inhibited the virus titer in the lungs of infected animals (Fig. 4). Arterial blood oxygen saturation levels were also significantly improved in DAS181-treated mice (not shown). Ribavirin, included as a positive control, also inhibited this virus infection, although not to the extent evoked by DAS181.

To evaluate therapeutic potential of DAS181 as a treatment for influenza, the fusion protein was applied at different time points relative to infection in the second mouse study. Treatments up to 48 h after virus exposure significantly prevented the death of the mice; all treatment regimens significantly reduced lung disease parameters (Table 5). A more stringent test of efficacy was conducted in the following study. Mice infected with IFV were treated by DAS181 once every other day beginning 48 h after virus exposure at dosages of 25 and 5 U/treatment. Significant protection by AvCD-AR was seen at 25 U/treatment.

We also tested the in vivo anti-IFV effect of the sialidase fusion protein in a ferret model which is thought to closely mimic human influenza (31, 42, 51). This experiment was performed with an earlier version of the fusion (DAS178, AR-AvCD). DAS178 differs from DAS181 in the location of AR tag (which caused lower yields in *E. coli*) but was otherwise indistinguishable in cell protection assays. In the vehicle-treated ferrets, virus shedding reached peak values on day 1 or 2 postchallenge, diminished over time, and became negative by

TABLE 2. Cell protection by DAS181 against recent clinical IFV isolates

Virus	Mean cell protection ± SD[a]	
	EC_{50} (nM)	EC_{90} (nM)
A/Singapore/35/2004 (H3N2)	0.6 ± 1.0*	4.5 ± 5.5†
A/Canada/600/2004 (H3N2)	0.1 ± 0.1‡	0.9 ± 0.7‡
A/Texas/05/2004 (H3N2)	0.2 ± 0.2‡	1.9 ± 2.3§
A/Hong Kong/2637/2004 (H1N1)	0.2 ± 0.7‖	26.5 ± 32.4‡
A/Hong Kong/2765/2004 (H1N1)	1.2 ± 1.3*	51.6 ± 69.5¶
B/Peru/1960/2004	0.2 ± 0.1†	0.5 ± 0.2†

[a] *, Six determinations; †, three determinations; ‡, five determinations; §, eight determinations; ‖, four determinations.

TABLE 3. Cell protection by DAS181 against IFV at various MOIs

Virus	EC_{50} (nM)[a] at MOI:			
	0.001	0.01	0.1	1
A/PR/8/34 (H1N1)	0.4	1.5	1.5	2.4*
A/Japan/305/57 (H2N2)	<0.1	<0.1	<0.1	5.9
A/Port Chalmers/1/73 (H3N2)	0.1	0.2	0.3	1.6
B/Maryland/1/59	<0.1	0.6	1.4*	ND
A/turkey/Wis/66 (H9N2)	<0.1	0.3	1.9	39.6
A/equine/1/56 (H7N7)	<0.1	0.3	0.4	5

[a] EC_{50} values were extrapolated from the cell protection assay dose-response curves as described in Materials and Methods, except that the MOI varied from 0.001 to 1. Each EC_{50} was measured in two independent experiments unless otherwise indicated. ND, not determined. *, Single experiment.

TABLE 4. Cell protection efficacy of DAS181 against A/PR/8/34 using various experimental protocols

EC_{50} (nM)[a]	Protocol	Description
1.7	P1	DAS181 for 2 h, add virus for 1 h, wash twice with PBS, add fresh medium, and incubate
2.7	P2	DAS181 for 2 h, add virus for 1 h, aspirate, add fresh medium, and incubate
0.1	P3	DAS181 for 2 h, add virus, and incubate
0.2	P4	DAS181 and immediately add virus and incubate
8.6	P5	Add virus for 1 h, add DAS181 for 2 h, aspirate, add fresh medium, and incubate
0.1	P6	Add virus for 1 h, add DAS181, and incubate

[a] EC_{50} values were extrapolated from cell protection assay dose response curves as described in Materials and Methods.

day 5 (Table 6). In contrast, in the group treated with the sialidase fusion protein, only 3 of 12 ferrets shed virus on day 1 postchallenge, and their nasal virus titers were about 100 times lower than those in the vehicle-treated animals. Three animals were completely protected against infection, signs of illness, and inflammatory response, as confirmed by a lack of seroconversion on day 14 postchallenge. The shedding in the remaining eight ferrets varied during the course of infection. However, in these animals, signs of inflammation in the nasal washes was reduced by about 40% (Fig. 5). It was also noticed that the 7-day treatment of DAS178 at 1,000 U/day (>1 mg/kg/day) did not cause any signs of toxicity or inflammation in ferrets that were not infected by the virus (Fig. 5).

DISCUSSION

The results of in vitro and in vivo studies have demonstrated the ability of a sialidase catalytic domain/AR GAG-binding sequence fusion protein to significantly inhibit the replication of IFVs, prevent influenza, or significantly reduce severity of the disease. By targeting the host cells rather than the virus, the sialidase fusion protein demonstrated distinct anti-IFV properties from the virus-targeting NAIs. In the MDCK cells, the state of cell surface desialylation sustained for at least 2 days after a single DAS181 treatment. Consistent with its long-lasting treatment effect, the subnanomolar EC_{50} of DAS181 was observed under an experimental condition in which the drug candidate was removed from the culture medium after the initial virus challenge (Table 1). In addition, DAS181 potency remained undiminished even when the cell treatment was performed 24 h prior to the virus challenge (not shown). This is in contrast to the NAIs: the EC_{50} of oseltamivir in-

TABLE 5. Intranasally administered DAS181 protected mice a from lethal dose of influenza A/NWS/33 (H1N1) virus

Study and compound, dosage/treatment[a]	Dosing time start[b]	Surv/total[c]	Mean day to death ± SD[d]	Mean day 11 % SaO_2 ± SD	Mean lung virus titer (log_{10}/g)	Mean day 6 lung wt (mg)
Study 1						
AvCD-AR, 60 U/treat	48 h pre	10/10***[g]	>21.0 ± 0.0***	87.2 ± 6.6***	1.7***[d]	130***
AvCD-AR, 30 U/treat	48 h pre	10/10***	>21.0 ± 0.0***	89.2 ± 4.3***	1.5***[d]	–[e]
AvCD-AR, 3 U/treat	48 h pre	6/6***	>21.0 ± 0.0***	85.2 ± 6.3***	3.0**[d]	205
AvCD-AR, 0.3 U/treat	48 h pre	7/7***	>21.0 ± 0.0***	84.6 ± 6.2***	2.6***[d]	120***
AvCD-AR, 0.03 U/treat	48 h pre	3/5**	13.0 ± 2.8**	82.6 ± 5.6***	5.4**[d]	190**
Ribavirin, 75 mg/kg	4 h pre	4/10**	9.8 ± 3.0	77.2 ± 3.2**	5.45**[d]	155***
Vehicle	48 h pre	0/19	8.9 ± 1.4	75.0 ± 0.0	6.75[d]	325
Normal control	48 h pre	–	–	91.0 ± 3.6	0.0[d]	115
Study 2						
AvCD-AR, 30 U/treat	48 h pre	11/11*	>21.0 ± 0.0***	89.2 ± 6.0**	1.8***[A]	126***
AvCD-AR, 30 U/treat	24 h pre	11/11*	>21.0 ± 0.0***	85.1 ± 4.9	2.0***[B]	154**
AvCD-AR, 30 U/treat	4 h post	11/11*	>21.0 ± 0.0***	91.0 ± 5.1***	1.8***[C]	158**
AvCD-AR, 30 U/treat	12 h post	11/11*	>21.0 ± 0.0***	87.7 ± 4.3**	1.45***[C]	174**
AvCD-AR, 30 U/treat	24 h post	11/11*	>21.0 ± 0.0***	85.7 ± 6.7	1.2***[C]	158**
AvCD-AR, 30 U/treat	48 h post	11/11*	>21.0 ± 0.0***	85.1 ± 6.1	1.0***[C]	198*
AvCD-AR, 30 U/treat	72 h post	9/11	9.0 ± 1.4	79.4 ± 3.1	2.0**[C]	210*
Ribavirin, 75 mg/kg	4 h pre	8/10	15.0 ± 0.8*	86.7 ± 7.1*	4.4[C]	164**
Vehicle	48 h post	12/21	9.6 ± 3.0	80.5 ± 6.3	4.4[C]	295
Normal control	–	–	–	90.0 ± 6.1	0.0[C]	118
Study 3						
AvCD-AR, 25 U/treat	48 h post	6/10**	8.8 ± 1.0***	81.3 ± 7.4**	5.4[D]	200*
AvCD-AR, 5 U/treat	48 h post	0/8	9.3 ± 1.2***	75.0 ± 0.0	5.6[D]	262
Ribavirin, 75 mg/kg	4 h pre	10/10***	>21.0 ± 0.0***	87.6 ± 5.2***	4.7**[f]	106***
Vehicle	–	0/18	7.2 ± 0.6	75.0 ± 0.0	6.2[f]	307
Normal control	–	–	–	90.4 ± 3.6	0.0[f]	115

[a] Study 1, twice-daily dosing for 7 days (14 doses total); study 2, once-daily dosing for 5 days (5 doses total); study 3, every-other-day dosing (3 doses total). treat, treatment.

[b] That is, before (pre) or after (post) viral challenge.

[c] Surv/total, number of survivors/total number of animals tested.

[d] Measurements were obtained on day 3 when the virus titer in the placebo group reached its peak.

[e] –, Insufficient numbers after day 3 due to anesthesia-related deaths.

[f] Superscripts A, B, and C: measurements taken on day 3 (A), 6 (B), or 9 (C) after viral challenge to reflect the treatment schedule. Superscript D: measurement taken on day 6 after viral challenge.

[g] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to vehicle [saline-treated controls]).

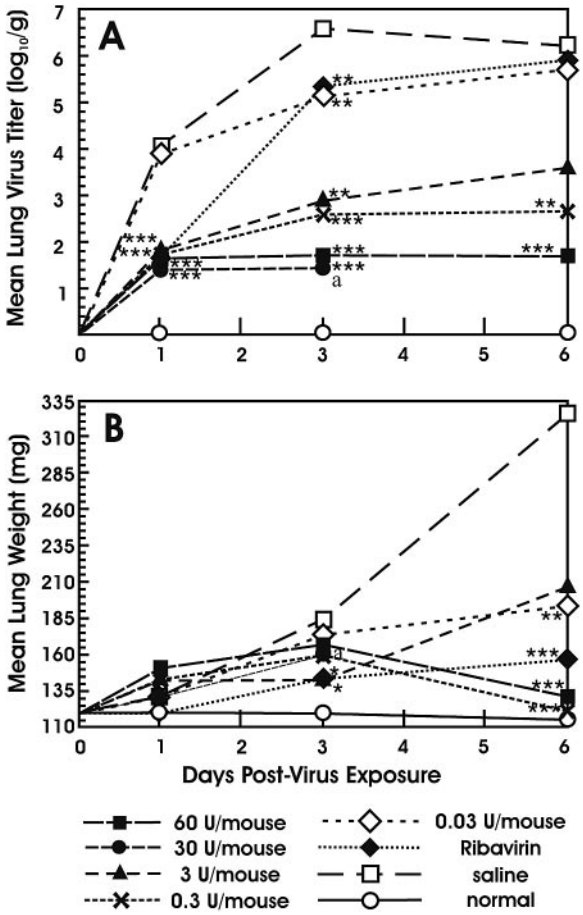

FIG. 4. Effects of DAS181 on lung virus titer (A) and lung weight (B) in infected mice. The results are part of experiment 1 in Table 5. The data at each time point were derived from three mice. Statistically significant values are labeled with one ($P < 0.05$), two ($P < 0.01$), or three ($P < 0.001$) asterisks. Ribavirin was used at 75 mg/kg.

creased 5,000 times (from 20 nM to 100 μM) when the compound was removed immediately prior to the virus infection (50). The efficacy of many antiviral compounds is influenced by the infectious dose of the virus; they are often more effective in cell culture at low MOIs and less active (or inactive) at higher virus-to-cell ratios. For the NAIs, such as oseltamivir and RWJ-270201, over the range of MOI 0.0002 to 0.02, every fivefold increase in MOI raised the EC_{50} of both compounds by 7 to 10 times (from 10 nM at an MOI of 0.0002 to >10 μM at an MOI of 0.02) (50). In contrast, DAS181 maintained a similar level of efficacy when the infectious dose was increased from an MOI of 0.001 to an MOI of 0.1 (Table 3).

Consistent with a previous report (52), 70 to 80% of the surface sialic acid was removed by DAS181 treatment (Fig. 2). Stray et al. reported that viral binding could occur in MDCK cells that were rid of about 70% of the cell surface sialic acid (52); however, in the sialidase-treated cells, multicycle amplification of IFVs was inhibited by 99 to 99.99% (52). Therefore, although enzymatic desialylation does not result in complete elimination of sialic acid, the viral infection that arises from residual sialic acid on the cell surface is negligible. Our own results fully corroborate the data of Stray et al. and demonstrate that it is unnecessary to completely eliminate cell surface sialic acid in order to achieve a desired therapeutic effect. In fact, further analysis of our in vitro results revealed that 50 to 70% cell surface sialic acid removal afforded >90% cell protection against all of the influenza viruses tested.

It was reported that cell surface sialic acids were primarily derived from glycoproteins (93%), and resialylation of the cell surface was mostly contingent upon de novo protein synthesis (41), even though at early time points a low level of sialic acid on the cell surface came from preexisting internal pools (41). Our observation on the surface sialic acid turnover after DAS181 treatment was consistent with this notion. A small but rapid rise in sialic acid level immediately after DAS181 treatment was most likely due to exchange with the internal pools of sialylated proteins (Fig. 2B). A slower return of sialic acid to the cell surface after the brief initial period probably reflected the rate of de novo protein synthesis that over time led to replenishment of sialylated proteins on the cell surface. The sialic acid turnover rate in the normal human respiratory epithelium is not known, but it is reasonable to expect that the sialic acid turnover is much slower in the normal respiratory epithelium than in the MDCK cells due to the lower cell

TABLE 6. Virus replication in the respiratory tract of DAS178 and vehicle-treated ferrets

Group and animal no.	Ferret tag no.	Virus titer (log_{10} $TCID_{50}$/ml) on day p.i.[a]:					Post challenge HI titers[c]
		1	2	3	4	5	
Vehicle-treated group							
1	228	5.7	4.2	4.2	1.7	–	640
2	784	3.9	4.9	1.9	1.9	–	640
3	793	4.4	4.2	2.4	3.9	–	640
4	794	4.9	5.9	1.4	–	–	160
5	789	4.4	4.2	3.4	3.4	–	640
6	799	3.7	4.4	3.4	–	–	320
7	811	4.4	4.4	–	–	–	1280
8	841	4.2	4.7	2.7	1.9	–	320
Mean[b]		4.4	4.7	2.7	3.7	–	
SD		0.4	0.7	1	0.4	–	
No. shed/total no.		8/8	8/8	7/8	5/8	0/8	
AR-AvCD-treated group							
1	780	–	–	–	NA	NA	NA
2	791	2.2	5.2	4.9	4.2	1.7	640
3	804	–	4.7	3.7	1.7	–	1280
4	803	–	–	–	–	–	≤01
5	805	–	–	–	–	–	≤01
6	806	–	–	–	–	–	≤01
7	810	2.2	4.7	3.2	2.9	–	160
8	812	–	–	4.4	–	–	640
9	813	–	3.2	4.4	4.7	–	160
10	819	2.7	5.2	–	–	–	320
11	828	–	4.9	1.9	1.7	–	320
12	843	–	4.4	4.9	4.9	3.4	320
Mean[b]		2.4	4.6	3.9	3.4	2.6	
SD		0.3	0.7	1.1	1.5	1.2	
No. shed/total no.		3/12	7/12	7/12	6/11	2/11	

[a] –, All nasal washes collected after day 5 postchallenge were negative for virus. Nasal washes recovered from the uninfected treated ferrets were negative for virus (not shown). NA, not applicable (the ferret died on day 4 postinfection due to an accident).

[b] That is, the mean value calculated for the ferrets that shed virus.

[c] HI, hemagglutination inhibition.

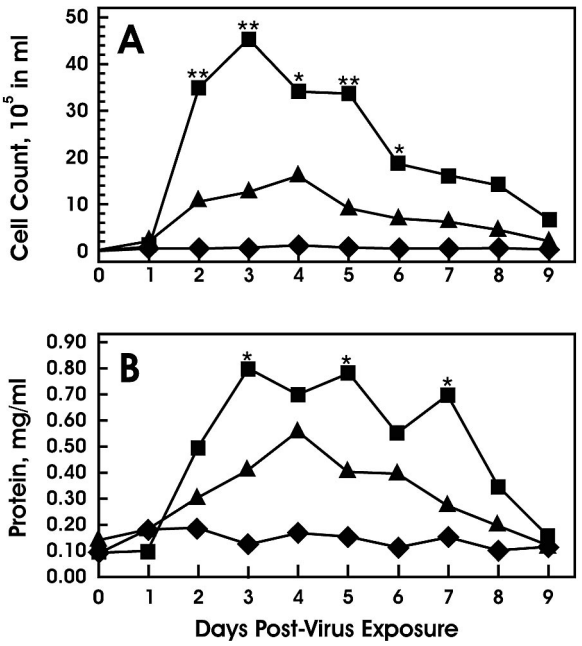

FIG. 5. Total inflammatory cell counts (A) and protein concentrations (B) in nasal washes from ferrets. Infected ferrets were vehicle treated (■) or were treated with DAS178 (▲). Uninfected animals were treated with DAS178 (◆) only. Statistically significant values are labeled with one ($P < 0.05$) or two ($P < 0.01$) asterisks. No that only the DAS178-treated ferrets that showed positive virus shedding were included in the analysis.

proliferation rate and slower protein synthesis in the differentiated epithelium. As a result, the treatment effect of DAS181 may last much longer than 2 days in the normal human airway as seen in the MDCK cells. Preliminary data with the HAE cultures indicated that desialylation by a single DAS181 treatment would last for more than 7 days in the respiratory epithelium (unpublished results). In spite of this reasoning, it should also be noted that faster sialic acid turnover may occur in the inflamed epithelia during influenza. Therefore, for influenza prophylaxis, infrequent dosing of DAS181 will likely be sufficient, whereas a relatively more frequent dosing regimen may be required for the treatment of an ongoing infection. It is, nevertheless, encouraging that once-every-other-day dosing of DAS181 resulted in significant therapeutic effects in mice even when the treatment was initiated at 48 h after virus infection (Table 5).

Generally, mice can only be infected by IFV strains that are previously adapted in mice, but ferrets can be infected with human unadapted influenza viruses and produce signs of upper respiratory tract diseases comparable to those seen in humans. Thus, ferrets are commonly used to evaluate influenza drug candidates (31, 42, 51). The lethal mouse model used in the present study represents influenza infection in the most severe form, whereas the self-limiting infection seen in the ferret model may mimic the more commonly encountered, milder form of influenza. Together, the two models cover a broad spectrum of influenza clinical manifestations. The ferret study reported here, however, was not optimal for evaluating the sialidase fusion protein candidate. To obtain full treatment effects, the sialidase fusion protein should evenly cover the surface of the upper and central respiratory tract, but this cannot be consistently achieved by the delivery method of nasal drops in larger animals such as ferrets. In spite of this, the sialidase fusion candidate still completely protected three animals as confirmed by negative seroconversion and significantly decreased the disease severity in the remaining eight ferrets. The animals also appeared to be more alert and active compared to the untreated ferrets that were invariably lethargic and feverish. Additional studies are being planned in which DAS181 will be formulated and delivered as dry powder inhalant, which is the designated delivery method in humans.

The sialic acids are negatively charged monosaccharides that are usually located at the outermost position of the polysaccharide chains attached to glycoproteins or glycolipids. Because of their negative charge, they may specifically or nonspecifically repel cell-cell interactions; they also serve as the ligands for various lectins (56). Selectins are the sialic acid-binding lectins that are found on blood cells or endothelial cells. It was proposed that interaction between sialic acids and selectins is involved in adhesion of leukocytes to the vascular endothelium (56). Another sialic acid-binding lectin is factor H that inhibits activation of the alternative complement pathway (35). Sialidase treatment of the cells has been shown to promote complement activation in vitro (14, 19, 33). However, in vivo, the apical surface of the airway epithelium does not have direct contact with the complement factors that are normally confined to the blood. It is therefore unclear what the physiological roles of sialic acids are on the airway surface other than serving as receptors for influenza virus, as well as parainfluenza virus (17).

Sialidase activities are normally present in many human tissues, including the salivary glands and the lungs (1); the in vivo effect of sialidase treatment has been previously evaluated in the context of airway hyperreactivity. *V. cholerae* sialidase was administered intratracheally in guinea pigs, and the sialidase treatment significantly reduced substance P-induced bronchoconstriction (21). Similar results were also obtained using *C. perfringens* sialidase and the isolated trachea and lungs from guinea pigs and rats. Again, sialidase treatment had no effect on smooth muscle contractions induced by acetylcholine, histamine, and 5-hydroxytryptamine, and it inhibited tracheal contraction induced by ovalbumin or compound 48/80 (22). Sialidase treatment did not change the rheological properties of respiratory mucus, nor did it affect the normal mucus transport activity on ciliated epithelium (25, 32). Extensive in vivo safety evaluations of DAS181 are being planned. Our current preliminary in vitro and in vivo data indicated a healthy safety margin for the drug candidate.

Cellular adhesion by some of the most important respiratory bacteria, including *Haemophilus influenzae* (10, 24, 55), *Streptococcus pneumoniae* (4), and *Pseudomonas aeruginosa* (3, 16, 28, 39, 40, 43), have been reported to be mediated by binding to the sialic acid receptors on the host cells. Sialidase treatment of human pharyngeal epithelial cells inhibited adherence by *H. influenzae* (24). Peltola et al. reported that mice infected with recombinant influenza viruses that carried higher neuraminidase (NA) activities were associated with a higher incidence of secondary pneumonia after inoculation with type 2 *S. pneumoniae* D39 strain. The authors hypothesized that desialylation by the viral NA may cause increased cell binding by *S. pneu-*

moniae (30, 36, 37). However, using a type 19 *S. pneumoniae* strain, Seki et al. failed to demonstrate potentiated secondary bacterial pneumonia following influenza virus infection unless the mice were chronically colonized with *P. aeruginosa* (45). In light of these conflicting reports, we performed several experiments in vitro to observe cell adhesion by different strains of *S. pneumoniae* and *H. influenzae* to A549 cells. Over a broad range of bacterial input, DAS181 treatment did not increase cell adhesion by four strains of *S. pneumoniae* (including the D39 strain), nor did it increase cell adhesion by three strains of *H. influenzae* (unpublished results). Further evaluations on this issue will be carried out in vivo.

The sialidase fusion protein DAS181 represents a novel, potentially broad-spectrum influenza therapeutic agent. Although DAS181 is designed to be nonimmunogenic to humans, immunogenicity remains an important issue to be evaluated by animal studies and clinical trials. By utilizing the *A. viscosus* sialidase that can effectively degrade receptor sialic acids for both human and avian IFVs, DAS181 potentially confers protection against a very broad range of influenza viruses, including the future pandemic viruses. It may also remain effective when viral strains change yearly. A recent report indicated that an IFV reassortant generated in the laboratory used a less common form of sialic acids, α(2,8)-linked sialic acid, as the receptor (58). Interestingly, *A. viscosus* sialidase can cleave the α(2,8)-linked sialic acid as well (54). Since DAS181 targets cellular receptors rather than a viral gene product, the chance of influenza viruses developing drug resistance is decreased. Besides serving as receptors for influenza virus, sialic acids are also used as receptors by parainfluenza virus, some bacteria, and bacterial toxins (57). Therefore, the potential therapeutic value of DAS181 may go beyond influenza prophylaxis and treatment and warrants further evaluation.

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